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Article

Nicotinic agonists promoted the activation of nicotinic acetylcholine α7 receptors (α7 nAChR) in neurons, but failed to activate these receptors in mouse peritoneal macrophages

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Abstract: Nicotinic acetylcholine receptor (nAChR) of subtypes said "neuronal" are expressed in epithelial and immune system cells and participate in acetylcholine signaling by neural or non-neural pathways. It has been shown in macrophages that activation of type α7 nAChRs inhibits the release of pro-inflammatory cytokines, but the ion channel function has not been recorded in these cells. The objective of this work was to clarify what are the molecular mechanisms of transduction of α7 nAChRs in macrophages. To this end, RAW 264.7 cells, mouse peritoneal macrophages and rat hippocampal neurons were used. Cells were submitted to electrophysiological studies and stimulated with brief applications of the agonists acetylcholine, choline and nicotine, associated or not with the allosteric modulator PNU-120596. Responses to ATP were recorded as a reference. Furthermore, macrophages were submitted to cytokine quantitation. The electrophysiological results showed that macrophages responded to ATP but did not show whole-cell current by stimulation with nicotinic agonists. However, hippocampal neurons stimulated in the same pharmacological conditions of the macrophages showed ionic currents typical of the α7 nicotinic receptors. No effect of nicotine was observed in the lipopolysaccharide-induced TNF-α release. These results suggest that the α7 nAChR in macrophages do not work as ion channels similar to those expressed in neurons.

Keywords: Nicotinic receptor, Macrophage, Electrophysiology, Immune cells, Raw 264-7cells

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1. Introduction

The nicotinic acetylcholine receptors α7 (α7 nAChRs) is one of the acetylcholine receptor subtypes that in neurons are located mainly at the presynaptic terminals, functioning as auto-receptors and hetero-receptors. In this way, they modulate the synthesis and release of acetylcholine (ACh) and other neurotransmitters [1-5]. Also, they may mediate rapid postsynaptic excitation in certain neurons, such as cortical and hippocampal interneurons [6-8] and supraoptic nucleus [9]. The α7 nAChRs are widely distributed in the central nervous system (CNS) and peripheral including dorsal root ganglia, bulb, hypothalamus, hippocampus, cortex [10-16] and seems to play an important role in synaptic plasticity, neuroprotection, neurite outgrowth and neurogenesis, neuronal differentiation [17] and nociception in the CNS and peripheral [18].

Nicotinic α7 receptors, like all other nAChRs, are permeable to Na^+, K^+, and Ca^{2+} cations and exhibit differences in their activation and deactivation kinetics. They are receptors with activation of rapid onset - with a maximum transient probability of channel opening of less than 1 ms, followed by rapid desensitization [3,12,19-21]. This rapid desensitization is an agonist concentration-dependent property that is intrinsic to the α7

receptor [22]. The α7 nAChRs are highly permeable to Ca^{2+} [23,24] more than Na^{+}, in a ratio of about 3-4:1 relative to most other nAChRs [10, 25]. Due to this factor, Albuquerque et al. (2009) suggest that the activation of this channel may influence Ca^{2+}-dependent mechanisms, including the activation of metabotropic pathways of second messengers [10]. In addition, there is evidence that Ca^{2+} driven by α7 nAChRs may play the role of amplifying intracellular Ca^{2+} levels, stimulating the release of stocks, and activating multiple Ca^{2+}-dependent kinases such as protein kinase C (PKC) [26].

It has been reported that α7 nAChRs are expressed in non-neuronal cells [12,27,28] such as cells of the immune system [3,29-34]. And, it appears that ACh released by non-neural cells acts in an autocrine and paracrine way through the nicotinic receptors expressed in these cells [35,36]. In addition, studies suggest that ACh through the cholinergic anti-inflammatory pathway participates in the anti-inflammatory effects by the activation of α7 nAChRs expressed in human macrophages [32,33,37-39].

The data indicate that both vagal mouse stimulation and administration of nicotinic agonists in human macrophages in vitro promotes a reduction in the production of proinflammatory cytokines by macrophages [32,39,40]. On the other hand, signals from the periphery, such as IL-1β release, are transmitted, via afferent fibers from the vagus nerve to the nucleus of the solitary tract (NTS) [41,42] and from this, through an interneuron, to the dorsal motor nucleus of the vagus (NMDV) where the vagal efferent neurons are located. This demonstrates that the efferent vagus nerve seems to participate in a neuroimmune connection with characteristics of a reflex arc [43,44].

Was also showed the expression of α7 nAChRs in RAW 264.7 cells, a murine macrophages cell line, Kupffer cells and peritoneal macrophages [34, 45-49]. The selective α7 nicotinic agonists, AR-R17779, GSK1345038A and nicotine (Nic) reduced the production of pro-inflammatory mediators and the activation of NF-κB in peritoneal macrophages [48,50,51]. Also, the administration of nicotine in LPS-stimulated peritoneal macrophages promoted reduced production of IL-6 and TNF-α by these cells [52]. And, in a study in which the effects of choline on TNF levels in LPS-challenged RAW 264.7 cells were observed, it was shown that this substance dose-dependently reduced the production of cytokines by these cells compared to control [53] It was verified that RAW 264.7 cells also express the α2, α5, β2 subunits of the nAChRs and the m1 and m3 mAChRs [54].

Alpha-7 nAChRs also mediate effects of nicotine on microglia, which are macrophages resident in central nervous tissue [29,55,56]. Shytle et al. (2004) showed that nicotine as well as ACh dose-dependent promotes a decrease in the release of TNF-α by microglia. This effect was blocked by both α-BGT and the non-competitive antagonist of nAChRs, mecamylamine [57]. These authors have shown a reduction in the activity of the protein phosphatase and mitogen-activated protein kinases (MAPK) MAPKp44/42 and p38 in these cells by stimulation with these substances. These kinases, MAP, p38 as well as extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) are involved in the synthesis of cytokines such as TNF-α [55]. This suggests that the anti-inflammatory effect of these substances occurs by the inhibition of these kinases.

Despite many signaling pathways have already been implicated downstream of the activation of α 7 nAChRs, in the cells of the immune system the mechanism of immediate response remains obscure, without knowing whether it involves ionic permeation or only metabotropic responses, perhaps mediated by allosteric interactions with other proteins. In addition, the activity of the α7 nAChRs channel in cells of the immune system has not yet been demonstrated by electrophysiological methods in the case of peritoneal macrophages.

The aim of this research was to clarify the molecular mechanisms of transduction of α7 nAChRs in macrophages, fundamental cells in the immune response and in the reaction to tissue damage using the same electrophysiological and pharmacological methods already validated in the study of α7 receptors in neurons.

2. Methods

2.1. Animals

Wistar rats and Swiss and C57BL/6 mice were used. The 8-week-old male mice (Swiss and C57BL / 6) weighed between 25 and 30g were supplied by the animal breeding division of the Microbiology Institute of the Federal University of Rio de Janeiro. Already the rats were created and kept by animal breeding division of Laboratory of molecular pharmacology - ICB/UFRJ. Rats were mated and after 16 to 18 days of gestation were sacrificed to the culture of hippocampal neurons.

The laboratory room temperature was maintained at 24 ± 2 °C with a light / dark cycle of 12 hours (lights lighting at 06:00). During this period the animals had free access to water and feed (CR-1, Nuvilab), being handled every 48 hours to clean the cages. Mice were individually maintained in the cages after treatment with thioglycollate (3-4 days). All the experimental procedures were conducted in compliance with the recommendations of the Ethics Commission for the use of experimental animals of the Federal University of Rio de Janeiro, RJ, Brazil.

2.2. Culture of mice peritoneal macrophages

Peritoneal macrophages were obtained as previously described [58]. Swiss mice received an i.p. dose of aged thioglycollate (2 ml; 3%) and 3-4 days later were sacrificed with CO_2. Then, 5 ml of frozen Roswell Park Memorial Institute culture medium (RPMI 1640) was injected into the animal's peritoneum to obtain the macrophages. The total medium harvested was centrifuged and the cells resuspended with the RPMI 1640 cell culture medium supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin and 100 μg/ml streptomycin - (both purchased from Gibco). This same medium was used as the medium of maintaining macrophages. After resuspension, cells were seeded in plastic Petri dishes (35 mm diameter) previously treated with poly-L-lysine for 24 h for electrophysiological experiments (5×10^5 cells in 2 ml culture medium) and incubated in the incubator at 37 °C and 5% CO_2. After 1 hour of incubation the medium was changed to the removal of the non-adherent cells. For some experiments, cultures were treated with addition of 100 ng/ml lipopolysaccharide (LPS, type O111: B4, Sigma-Aldrich) for 4 and 24 hours.

2.3. Culture of RAW 264.7 cell

RAW 264.7 cells were obtained from the Cell Bank of the State of Rio de Janeiro and maintained in T25 cell culture bottles in cell culture medium (RPMI 1640 supplemented with 10% FBS, and 100 U/ml penicillin and 100 μg /mL streptomycin - (both from Gibco)). Cultures were maintained at 37ºC, 5% CO_2. For the electrophysiology experiments, they were seeded in plastic petri dishes, 35 mm in diameter, previously treated with poly-L-lysine per 24 hours. RAW 264.7 cells were investigated in the presence and absence of LPS for 4 and 24 hours.

2.4. Culture of hippocampal neurons

Culture of hippocampal neurons was performed from fetuses of rats with E16 to E18 as previously described [59]. Pregnant rats were sacrificed with CO_2 and the culture of hippocampal neurons of fetuses from 16 to 18 days of embryonic life (E16 and E18) was realized. Hippocampus were desiccated and transferred to a petri dish where they were treated with trypsin (0, 25%) for 25-35 minutes and incubated at 37 °C and 5% CO_2. The entire procedure from cesarean section and trypsinization was performed for a time period of 50 to 60 minutes.

After trypsinization, hippocampal tissues were washed 3 times and dissociated in 10/10 neuron culture medium (Minimal Essential Medium (MEM) supplemented with 10% FBS, 10% inactivated horse serum (HS), sodium bicarbonate ($NaHCO_3$, 44 mM), DNAse

type II (20 μg/ml) and glutamine (1 mM)). After this procedure, the cells were resuspended to a final dilution of one hippocampus per ml of cells and the plating was performed by adding 1 ml of 10/10 medium with cells and 1 ml of 10/10 cell free medium per dish. These 35 mm petri dishes were pretreated with poly-L-lysine for 24 h.

On the day after plating the cells, the plating medium was replaced by 10 neuron culture medium (MEM supplemented with 10% HS, glutamine (1 mM) and $NaHCO_3$ (44 mM), which was used as the medium of maintaining the neurons. After 6 days of culture, 200 μl of the antimitotic agent FDU/U (5-fluoro-2-deoxyuridine (FDU; 8.1 mM) (2 mg/ml), uridine (U; 4,1 mM) (1 mg/ml)) were added. After 24 h, the medium was again exchanged for 10 medium and from this day the exchanges of the medium were intercalated for 1 to 2 days until the culture was no longer viable.

2.5. Electrophysiology

The electrophysiological recordings were performed in systems that include patch-clamp amplifiers, inverted microscopes, micromanipulators, stimulators, microperfusion system (U-tube) with pulse application of up to 2 seconds [60], A/D interface, software and microcomputer, optimized for registration of α7 nAChRs mediated fast currents in neurons [61]. The analog output signal from the amplifier was filtered at 2 KHZ by an 8-pin Bessel filter (Frequency Devices, USA) and scanned at 4 KHz through a TL-1 digital-analogue converter (Axon instruments, USA). The data were acquired for the computer by the Clampex 6 program (Axon Instruments, USA) and the records of ion currents were made through the whole-cell patch clamp technique [62].

In order to obtain the record of currents of whole cell, the cell culture plates were removed from the incubator and the culture medium was replaced with extracellular physiological solution (SE; 165 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 10 mM dextrose, 5 mM HEPES). The pH of SE was adjusted to 7.35 with NaOH (~2 mM) and the plates were then placed on the inverted microscope.

Cell ion current uptake was performed using a borosilicate glass micropipettes (World Precision Instruments, USA) made with a handle (Sutter Instruments, USA). The micropipettes were filled with intracellular physiological solution (SI; 80 mM CsCl; 80 mM CsF; 22.5 mM CsOH; 10 mM ethylene glycol-bis (β-aminoethyl ether)-N, N, N', N' tetraacetic acid (EGTA); 5 mM 2- [4- (2-hydroxyethyl) -1-piperazinylethanesulfonic acid (HEPES)). The pH of SI was adjusted to 7.35 with CsOH (~ 3.5 mM). Then it was inserted into the micropipettes a chlorinated silver wire which was connected to the micro amplifier. The resistance of the microelectrodes remained throughout the study between 2.5 and 6 MΩ. After the formation of the seal, the transmembrane potential was adjusted to - 60 mV. Then, the current from the stimulation of the cells by the substances of interest with the U-tube microperfusion system was recorded. This current was observed and recorded through the Clampex 6 computer program (Axon Instruments, USA). All experiments were performed at room temperature (21-23 ° C).

2.6. Substances and concentrations administered during electrophysiological studies

Neurons, peritoneal macrophages and RAW 264.7 cells were stimulated with the nicotinic agonists acetylcholine (ACh; 1 mM), nicotine (Nic, 1 mM and 100 μM) and choline (Col; 10 mM) in the presence or absence of the positive allosteric modulator of type II selective for α7 nAChRs, PNU-120596 (10 μM). The choice of this was because it promotes the destabilization of α7 receptor desensitization states [63-65].

As a control solution (CNTL) was used the SE and as a positive control to investigate the viability of macrophages was used sodium adenosine triphosphate (ATP-Na^+).

2.7. Data analysis

The maximum amplitudes of the currents recorded in the electrophysiological experiments were analyzed using the Clampfit program (Axon Instruments, USA). The mean values, DP and EPM were computed in MS-Excel spreadsheets.

3. Results

3.1. Immediate effects of ACh and ATP-Na⁺ on peritoneal macrophages

Analysis of ionic currents records detected by whole-cell patch clamp technique showed that, of the 47 cells investigated, 38 responded to ATP-Na⁺ (1 mM) (Figure 1B and 1D) and none to ACh (1 mM) (Figure 1A and 1C), both responses being tested in each cell. Nine cells did not respond to both substances (Table 1). Therefore, peritoneal macrophages stimulated with ACh pulses did not show ionic currents typical of the activation of nicotinic receptors in other cells. This result also excludes a muscarinic response coupled to ion channels in macrophages, at least on the relatively short time scale used in the registries.

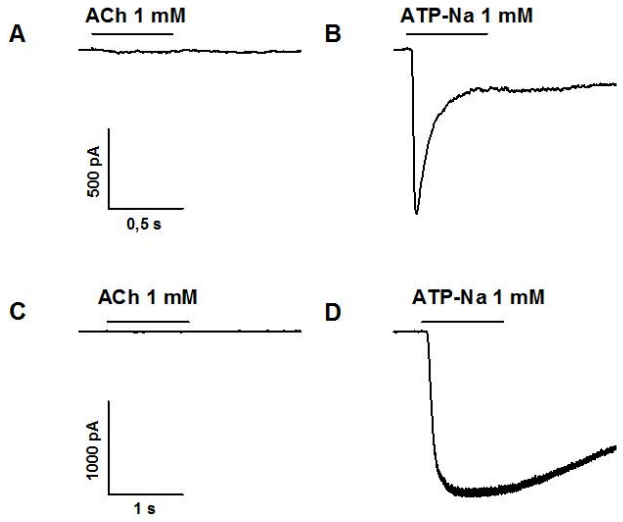

Figure 1. Examples of ionic currents in peritoneal macrophages. ACh (1 mM) and ATP-Na⁺ (1 mM) agonists were applied in 0.5 s (A and B) or 1 s (C and D) pulses, as indicated by the horizontal bars. A and B represent current traced obtained in sequence in the same cell, which did not respond to ACh, but responded to ATP-Na⁺ with a fast current with clear desensitization in the presence of the agonist. C and D represent another cell, which presented a different response to ATP, without desensitization. Cells were maintained at a potential of - 60 mV; representative records of 47 cells from 7 culture plates of 6 animals.

Table 1. Number of peritoneal macrophages that responded to stimulation with ATP-Na⁺ (1 mM) and with ACh (1 mM) by the whole cell patch clamp technique. (+): Presence of ionic current greater than basal noise.

ATP 1 mM	ACh 1 mM	
	+	–
+	0	38
–	0	9

Of the 38 investigated cells responding to ATP, seven appeared to exhibit rapid desensitization responses (Figure 1B) characteristic of the P2X1 and P2X3 receptors and 31 appeared to show slow desensitization responses (Figure 1D) typical of P2X2, P2X4 and P2X7 receptors.

3.2. Immediate effects of ACh and nicotine and the modulator PNU-120596 on hippocampal neurons

Considering the negative results of ACh tests in peritoneal macrophages, we decided to check whether the stimulus was adequate to evoke the response of the $\alpha 7$ nicotinic receptor and whether we could amplify the response to increase the sensitivity of the assay investigating nicotinic responses in hippocampal neurons.

The analysis of the currents detected with the whole-cell patch clamp technique in neurons allowed the validation of the system to the register the activity of $\alpha 7$ nicotinic receptors, as shown in Figure 2. The stimulation of ACh (1 mM) induced typical ionic currents of $\alpha 7$ nAChR, with rapid activation and complete desensitization in a fraction of a second (Figure 2A). This response was greatly enhanced by the association with the positive allosteric modulator type II, PNU-120596 (10 μM), even with a 10-fold lower concentration of ACh (100 μM) (Figure 2B). The current presented an increase in its amplitude as well as a pronounced prolongation of the activation, without signs of desensitization. These same typical responses were observed in cells stimulated with 10 mM choline (Figure 2C) and choline (1 mM) associated with PNU-120596 (10 μM) (Figure 2D). Amplification of the allosteric modulator response was also observed with nicotine (0,1 mM) associated with PNU-120596 (10 μM), even when compared to the response to a saturating concentration of nicotine (1 mM) in the same cells (Data not shown).

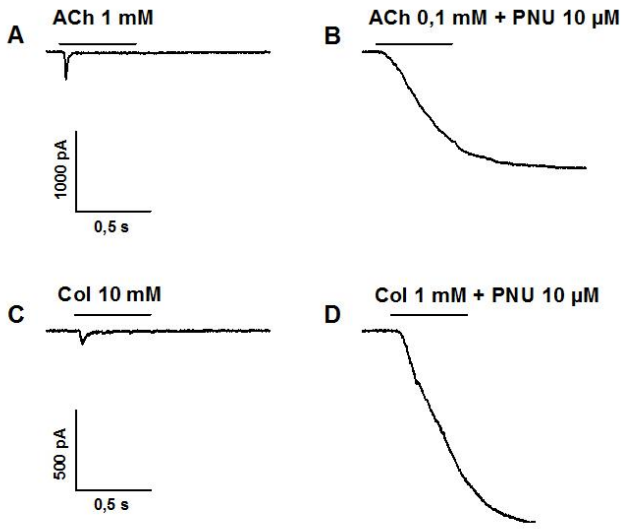

Figure 2. Examples of nicotinic ionic current in rat hippocampal neurons. ACh (1 mM and 0,1 mM, A and B) and choline (1 mM and 0,1 mM, C and D) were applied with or without PNU-120596 (10 μM) in 0.5 s pulses, as indicated by the horizontal bar. Cells were maintained at a potential of - 60 mV.

Based on these results, was decided to use the agonists at submaximal concentrations in association with the PNU-120596 modulator at 10 μM for further testing on peritoneal macrophages.

3.3. Test of immediate response to α7 receptor activation in peritoneal macrophages

Peritoneal macrophages were stimulated with the ACh and choline endogenous agonists (selective for $\alpha 7$) and with nicotine alone or associated with the PNU-120596 modulator. Each cell was also stimulated with ATP-Na^+, about two minutes after the cholinergic stimulus.

Acetylcholine was evaluated in 21 cells. Of these cells, 17 responded to ATP-Na^+ (1 mM) (Figure 3B) and none to ACh (100 μM) associated with PNU-120596 (10 μM) (Figure 3A), both responses being tested in each cell. Four cells did not respond to both substances (Table 2). Already choline was investigated in 22 cells. Of these cells, 21 responded to ATP-Na^+ (1 mM) (Figure 4C; Table 3) and none to choline (10 mM) (Figure 4A) or to choline (1 mM) associated with PNU-120596 (10 μM) (Figure 4B), with the three responses being tested in each cell. And Nicotine was evaluated in seventeen cells. Of these cells, all responded to ATP-Na^+ (1 mM) (Table 3) and none to nicotine alone (100 μM); or nicotine (100 μM) associated with PNU-120596 (10 μM), the three responses being tested in each cell (Data not shown).

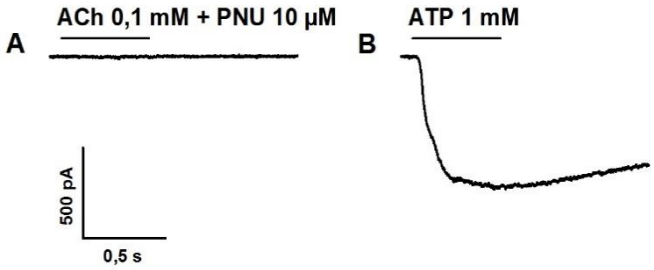

Figure 3. Examples of ionic currents in peritoneal macrophages. ACh (0,1 mM) associated with PNU-120596 (10 μM) and ATP-Na^+ (1 mM) were applied in 0.5 s pulses as indicated by the horizontal bar. The cell was maintained at a potential of - 60 mV; representative records of 21 cells from 5 culture plates of 4 animals.

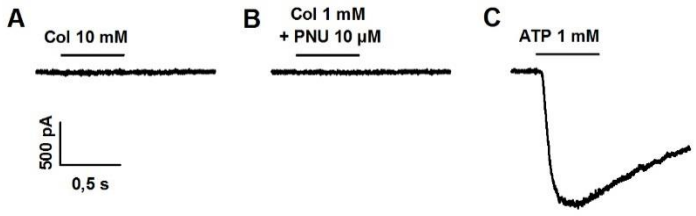

Figure 4. Examples of ionic currents in peritoneal macrophages. Choline (Col; 10 mM), Col (1 mM) associated with PNU-120596 (10 μM) and ATP-Na^+ (1 mM) were applied in 0.5 s pulses, as indicated by the horizontal bar. The cell was maintained at a potential of - 60 mV; representative records of 22 cells from 3 culture plates of 2 animals.

Table 2. Number of peritoneal macrophages that responded to stimulation with ATP-Na^+ (1 mM) and with ACh (100 μM) associated with PNU-120596 (10 μM) by the whole cell patch clamp technique. (+): Presence of ionic current greater than basal noise.

ATP 1 mM	ACh 0,1 mM + PNU 10 μM	
	+	–
+	0	17
–	0	4

Table 3. Number of peritoneal macrophages that responded to stimulation with ATP-Na^+ (1 mM), with Choline (Col; 10 mM), with Nicotine (Nic; 0,1 mM) and with Col and Nic (1 mM and 100 μM respectively) associated with PNU-120596 (10 μM) by the whole cell patch clamp technique. (+): Presence of ionic current greater than basal noise.

ATP 1 mM	Col 10 mM or Col 0.1 mM + PNU 10 μM		Nic 0,1 mM or Nic 0,1 mM + PNU 10 μM	
	+	–	+	+
+	0	21	0	17
–	0	1	0	0

3.4. Testing the immediate response to alpha7 receptor activation in RAW 264.7 cells

According to the literature, RAW 264.7 cells express $\alpha 7$ nAChR and activation of these receptors inhibits LPS-induced TNF-α release [48,53,66,67]. However, there is no report of an attempt to record ionic currents mediated by the $\alpha 7$ receptor in these cells. Thus, we investigated the immediate response of RAW 264.7 cells in the same way as we done with peritoneal macrophages.

The analysis of currents detected with the whole-cell patch clamp technique showed that, of the 25 cells investigated, 20 responded to ATP-Na+ (1 mM) (Figure 5C; Table 4) and none to ACh (1 mM) (Figure 5A); or to ACh (100 μM) associated with PNU-120596 (10 μM) (Figure 5B), with the three responses tested in each cell.

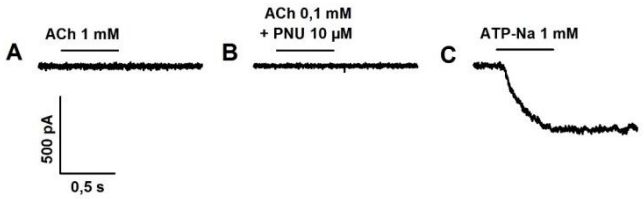

Figure 5. Examples of ionic currents in RAW 264.7 cells. ACh (1 mM), ACh (0,1 mM) associated with PNU-120596 (10 μM) and ATP-Na^+ (1 mM) were applied in 0.5 s pulses, as indicated by the horizontal bar. The cell was maintained at a potential of - 60 mV; representative records of 25 cells from 4 culture plates of 2 animals.

Table 4. Number of RAW 264.7 cells that responded to stimulation with ATP-Na^+ (1 mM), with acetylcholine (ACh; 1 mM) and with ACh (100 μM) associated with PNU-120596 (10 μM) by the whole cell patch clamp technique. (+): Presence of ionic current greater than basal noise.

ATP 1 mM	ACh 1 mM or ACh 0,1 mM + PNU 10 μM	
	+	–
+	0	20
–	0	5

3.5. Test of immediate response to α7 receptor activation in LPS pre-stimulated peritoneal macrophages

Having been observed an absence of immediate response to the different *α7* nAChR stimuli in the peritoneal macrophages, it was considered the hypothesis that a previous stimulus would be necessary to promote the expression of the receptors or their insertion in the cells membrane. It was then evaluated the effect of macrophages pre-incubation with LPS (100 ng/ml) for 4 or 24 h prior to electrophysiological records, again testing the response to the same agonist and modulator combinations from the previous experiments. It was initially tested ACh with the modulator in cultures pretreated with LPS for 4 h. Of the 23 cells investigated with the whole-cell patch clamp technique, all responded to ATP-Na^+ (1 mM) (Table 5) and none to ACh (100 μM) associated with PNU-120596 (10 μM), both responses being tested in each cell (Data not shown).

Table 5. Number of peritoneal macrophages stimulated with LPS for 4 h that responded to stimulation with ATP-Na^+ (1 mM) and with acetylcholine (ACh; 100 μM) associated with PNU-120596 (10 μM) by the whole cell patch clamp technique. (+): Presence of ionic current greater than basal noise.

ATP 1 mM	ACh 0,1 mM + PNU 10 μM	
	+	–
+	0	23
–	0	0

It was then tested a longer pre-incubation time with LPS using the three-pulse stimulus protocol for recording ion currents. In cultures pretreated with LPS for 24 h, of the 20 cells investigated, all responded to ATP-Na+ (1 mM) (Figure 6C; Table 6) and none to ACh (1 mM) (Figure 6A); or ACh (100 μM) associated with PNU-120596 (10 μM) (Figure 6B), the three responses being tested in each cell.

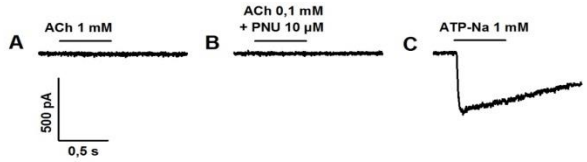

Figure 6. Examples of ionic currents in peritoneal macrophages stimulated with LPS for 24 h. ACh (1 mM), ACh (0,1 mM) associated with PNU-120596 (10 μM) and ATP-Na^+ (1 mM) were applied in 0.5 s pulses, as indicated by the horizontal bar. The cell was maintained at a potential of - 60 mV; representative registers of 20 cells of 4 culture plates of 4 animals.

Table 6. Number of peritoneal macrophages stimulated with LPS for 24 h that responded to stimulation with ATP-Na^+ (1 mM), with acetylcholine (ACh; 1 mM) and with ACh and Choline (100 μM and 1 mM respectively) associated with PNU-120596 (10 μM) using the whole cell patch clamp technique. (+): Presence of ionic current greater than basal noise.

ATP 1 mM	ACh 1 mM or ACh 0,1 mM + PNU 10 μM		Col 1 mM + PNU 10 μM	
	+	–	+	–
+	0	20	0	9

–	0	0	0	0

In the choline tests, of the 9 cells investigated, pretreated with 24 h LPS, all responded to ATP-Na^+ (1 mM) (Table 6) and none to choline (1 mM) associated with PNU-120596 (10 μM), the two responses being tested in each cell (Data not shown).

In nicotine tests in peritoneal macrophages pretreated with LPS for 24 h, of the 19 cells investigated, 18 responded to ATP-Na^+ (1 mM) and none to nicotine (100 μM), or to nicotine (100 μM) associated with PNU-120596 (10 μM), the three responses being tested in each cell (Data not shown).

4. Discussion

The data obtained in this work show that the stimulation with ACh, choline and nicotine agonists in primary cultured macrophages and RAW 264.7 immortalized cells did not promote a transmembrane ion current in these cells by the whole-cell patch clamp technique. They only responded to ATP-Na^+.

The response of peritoneal macrophages in this study was similar to that reported by Mikulski et al. (2010) in alveolar macrophages [68]. These authors showed that PC12 cells, which densely express $\alpha 7$ nicotinic receptors, readily responded to ACh (0,1 mM) whereas that rat alveolar macrophages showed no current alteration in response to this agonist but responded to ATP-Na^+ (0,2 mM). Similarly, Suzuki et al. (2006) showed that microglia, resident macrophages of the CNS, did not present a response to nicotine (100 μM) by the whole-cell patch clamp technique, but responded to ATP-Na^+ (100 μM) [55]. The experimental conditions used by Mikulski et al. (2010) [68] may have made it difficult to detect $\alpha 7$ response, because the agonist has been applied slowly, which may cause more desensitization than receptor activation [68].

The literature describes that extracellular ATP promotes the channel opening of several types of P2X receptors. These receptors, which are ligands-dependent ionic channels, belong to a family of purinergic receptors that are expressed in a wide variety of animal tissues and in many cell types, including lymphocytes and macrophages [69-71]. It has been reported that among the P2X1-7 receptors, the P2X4 and P2X7 receptors are the most prominent types of P2X receptors expressed in mouse peritoneal macrophages [69,70].

The opening time of P2X channels depends on the composition of the receptor subunit. For example, the P2X1 and P2X3 receptors desensitize rapidly in the continuous presence of ATP, whereas that the P2X2, P2X4 and P2X7 receptors are slowly desensitized [70,71]. The ATP responses obtained in this study showed that some cells exhibit rapid desensitization responses, characteristics of P2X1 and P2X3 receptors (e.g., Figure 1B), and others appear to have slow desensitization responses, typical of P2X2, P2X4 and P2X7 receptors (e.g., Figure 1D). These data also showed an adequacy of our system for the electrophysiological recording of membrane cationic currents in macrophages.

In addition, an adequacy of our system was observed for the recording of $\alpha 7$ receptor responses by the analysis of electrophysiological records in hippocampal neurons (Figures 2 and 3). The data then showed typical ionic currents of $\alpha 7$ receptors by stimulation of these cells with the same pharmacological conditions used in the experiments with macrophages. In addition, was observed a robust response in neurons with the co-application of the allosteric selective modulator, PNU-120596.

Studies have shown that the allosteric modulator PNU-120596 associated with $\alpha 7$ receptor agonists such as choline and acetylcholine, but not alone, potentiates the activation of individual ion channels, which can lead to an increase of up to 100,000 times in the probability of opening [21,64,71,72].

In this study, stimulation with ACh (1 mM) induced fast-activating ionic currents and desensitization also rapid, in fraction of seconds, typical of nAChR $\alpha 7$ in neurons

(Figure 2A), and these responses were greatly enhanced by the association with PNU-120596 (10 μM; Figure 2B), even stimulating with a 10-fold lower concentration of ACh (100 μM). The current presented an increase in its amplitude, as well as a pronounced prolongation of the activation, without signs of desensitization. These same responses were observed in cells stimulated with choline (10 mM; Figure 2C) and nicotine (1 mM and 0,1 mM; Figures 3A and 3C) alone or in association with PNU-120596 (10 μM) (Data not shown). Responses to agonists and agonists associated with PNU-120596 were observed in all tested neurons.

Based on this data, we investigated the effect of ACh associated with PNU-120596 in order to potentiate a possible response of the α7 receptor in macrophages. However, analysis of the currents records detected by whole-cell patch clamp technique showed that none of the peritoneal macrophages tested responded to these substances (Figure 3), nor to the full and selective α7 nicotinic receptor agonist, choline (Figure 4), or to nicotine associated with PNU-120596 (Data not shown). However, most of the cells tested responded to ATP-Na^+. These same conditions were also tested on Raw 264-7 cells. Figure 5 exemplifies the currents records detected by whole-cell patch clamp technique in a Raw 264-7 cell stimulated with ACh. However, we did not observed response of these cells to agonists, only to ATP-Na^+ (Data not shown).

According to Nizri et al. (2009), the expression of α7 receptors is over-regulated after activation of the immune system [73]. The literature reports that the reduction of proinflammatory cytokines by macrophages occur dependently on α7 nAChRs after induction of endotoxemia in animals [32]. A similar phenomenon can be observed in vitro, because anti-inflammatory effects of nicotine and other α7 nAChRs agonists have been observed in culture of immune cells pretreated with LPS.

In light of these data from the literature and the findings in this study we have tested the hypothesis that the expression or insertion of α7 nAChRs on the surfaces of immune cells depended on a previous stimulation, for example, LPS. Therefore, we investigated the effect of ACh associated with PNU-120596 on macrophages previously stimulated with LPS. However, in this study, it was not detect an ionic current in cells previously stimulated with LPS for 4 h (Data not shown) or 24 h, applying Ach (Figure 6), choline and nicotine both in peritoneal macrophages and in lineage of murine macrophages, RAW 264.7 (Data not shown).

The literature suggests that macrophage stimulated with nicotinic agonists previously challenged by LPS for a period of 4 h and 24 h induces an anti-inflammatory effect. Parrish et al. (2008) in their work demonstrated that choline (10 mM and 50 mM) promoted a reduction in the release of proinflammatory cytokines by RAW 264.7 cells submitted to LPS (4 ng/ml) for a period of 4 h. The same authors observed also a reduction of proinflammatory cytokines released by peritoneal macrophages submitted to LPS (100 ng/ml) for a period of 4 h [53]. In addition, a reduction of TNF-α production by stimulation with ACh (100 μM) was shown in peritoneal macrophages previously challenged with LPS for 24 h. In contrast, no change in TNF-α levels was observed in macrophages of α7-receptor knockout mice [66]. Tsoyi et al. (2011) showed a reduction of TNF-α cytokine and nicotine-induced HMGB from RAW 264.7 cells previously stimulated with LPS for 24 h [74] and these researchers suggest that these effects are mediated by α7 nAChRs. The immunomodulatory effect mediated by the α7 receptor in vivo has also been shown in models of LPS-induced endotoxemia [32,66]. However, it was not observed in this study whole-cell current in the patch clamp experiments in primary cultured macrophages and immortalized RAW 264.7 cells pre-stimulated with LPS, even using the potentiator PNU-120596.

Even though we observed no response in two cell types and with pre-treatment with LPS, we can not exclude the possibility that the channel responses can be observed in other experimental conditions. For example, it would be interesting to test the responses in the cell attached configuration, thus avoiding a disturbance of the intracellular environment,

which may affect receptor activity. Likewise, the experiments were performed at room temperature and this may have affected the functionality of the receptor. Thus, it would be interesting to perform the experiments at 37 °C.

PNU-120596 showed, in this study, to induce an important influx of ions through α7 nAChR into neurons. However, Thomsen and Mikkelsen, when investigating an activity of PNU-120596 associated with GTS-21 and other selective agonists of α7 nAChRs in the production of cytokines in microglia, showed that these substances did not promote a change in TNF-α levels released by these cells when stimulated by LPS [56]. This suggests that it is not the influx of ions through this receptor that is responsible for the ability of nicotine to induce an anti-inflammatory process in immune cells.

Shytle and colleagues have suggested that the activity of α7 nAChR involved in the anti-inflammatory effects depends on the activation of PLC, MAPKp44/42 and p38 in microglia [57]. These researchers reported a reduction in the activity of these enzymes by stimulation with nicotine and ACh in microglia submitted to LPS. These MAPK and p38 kinases, as well as ERK and JNK, are involved in the synthesis of cytokines such as TNF-α [55]. In addition, another study demonstrated that the activity of α7 nAChR prevents the tyrosine phosphorylation of STAT3 and that the inhibition of this protein reduces the production of proinflammatory cytokines [48,75]. This data contradicts previous work by the same group suggesting that the reduction of production of TNF-α by stimulated macrophages with nicotine requires the activation of the STAT3 pathway [76], but in both cases modulation of pathway is attributed to the association of nAChR with intracellular kinases, such as Jak2. This set of workers supports the hypothesis that α7 nAChR in immune cells are unconventional nicotinic receptors, coupled to G protein or other metabotropic mechanism. However, it is possible to reconcile this hypothesis with a conventional mechanism of activation if the α7 receptor is able to mediate increase of cytoplasmic calcium in immune cells according to Albuquerque et al. (2009) [10]. These authors suggest that the activation of this channel may influence Ca^{2+}-dependent mechanisms, including the activation of second messenger pathways. In addition, Uteshev (2012) indicates that Ca^{2+} driven by α7 nAChR may be involved in the activation of multiple Ca^{2+} -dependent kinases such as protein kinase C (PKC) [26].

The set of available evidence indicates that the mechanism by which α7 nAChRs modulate the release of cytokines is complex and probably involves steps not yet discovered. Thus, discrepancies in the literature may be related to uncontrolled and even unknown experimental variables.

5. Conclusion

In conclusion, based on the results presented, α7-nAChRs expressed in macrophages appear not to participate as ion channels of the anti-inflammatory effects resulting from the administration of nicotinic agonists. Finally, this study represents a commitment to our understanding of α7 nAChRs expressed on immune cells and their involvement in the cholinergic anti-inflammatory pathway. From this, other studies, such as more sensitive electrophysiological experiments or biochemical experiments on intracellular signaling pathways may provide new subsidies for science.

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